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MINIREVIEW

The Gut Microbiota of Laying Hens and Its Manipulation with Prebiotics and Probiotics To Enhance Gut Health and Food Safety

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ABSTRACT The microbiota plays a vital role in maintaining gut health and influences the overall performance of chickens. Most gut microbiota-related studies have been performed in broilers, which have different microbial communities compared to those of layers. The normal gut microbiota of laying chickens is dominated by *Proteobacteria*, *Firmicutes*, *Bacteroidetes*, *Fusobacteria*, and *Actinobacteria* at the phylum level. The composition of the gut microbiota changes with chicken age, genotype, and production system. The metabolites of gut microbiota, such as short-chain fatty acids, indole, tryptamine, vitamins, and bacteriocins, are involved in host-microbiota cross talk, maintenance of barrier function, and immune homeostasis. Resident gut microbiota members also limit and control the colonization of foodborne pathogens. In-feed supplementations of prebiotics and probiotics strengthen the gut microbiota for improved host performance and colonization resistance to gut pathogens, such as *Salmonella* and *Campylobacter*. The mechanisms of action of prebiotics and probiotics come through the production of organic acids, activation of the host immune system, and production of antimicrobial agents. Probiotic candidates, including *Lactobacillus*, *Bifidobacterium*, *Bacillus*, *Saccharomyces*, and *Faecalibacterium* isolates, have shown promising results toward enhancing food safety and gut health. Additionally, a range of complex carbohydrates, including mannose oligosaccharides, fructo-oligosaccharides, and galacto-oligosaccharides, and inulin are promising candidates for improving gut health. Here, we review the potential roles of prebiotics and probiotics in the reshaping of the gut microbiota of layer chickens to enhance gut health and food safety.

KEYWORDS gut microbiota, laying chicken, enteropathogens, feed supplements, gut metabolites

The gut microbiota is a complex community of hundreds of diverse microorganisms. The gut microbiota influences the host, playing a role in the modulation of the immune system, nutrient digestion, and regulation of intestinal function. These modulatory effects are mediated by the complex microbial interactions and metabolites produced by the microbial community members or derived from the transformation of host molecules or diet (1, 2). The microbial metabolites involved in host and microbiota cross talk include short-chain fatty acids (SCFAs), tryptamine, conjugated linoleic acids, indole and its derivatives, and bile acids transformed by the gut microbiota (1). Broilers and layers have different genotypes, very different lifespans in normal commercial production, and are reared in different conditions with different dietary requirements. Therefore, the composition of the gut microbiota in these two lines is different (3). The microbiota differences can differentially influence bird responses to stimuli and chal-

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lenges. For example, differences in the gut immune response to *Campylobacter jejuni* have been correlated with different patterns of microbiota composition between broilers and layers (2). The laying chicken gut microbial communities are influenced by multiple factors, such as flock age, production system, disease, diet, and antibiotics (4).

The gut microbiota composition can be enhanced and strengthened with the use of prebiotic and probiotic supplements in the feed. Prebiotics are most commonly complex oligosaccharides that are not digested by host enzymes and, hence, end up in the lower gut where they promote the growth and multiplication of resident gut microbiota. Therefore, prebiotics are fed to enhance the growth of beneficial resident gut bacteria. Examples of prebiotics are inulin, galacto-oligosaccharides, fructo-oligosaccharides, xylo-oligosaccharides, pectin, beta-glucans, and resistant starch. Probiotics are viable bacteria that, when delivered in sufficient quantity, can improve host health. Apart from a range of mostly Gram-positive bacteria, some yeasts and molds have also been used as probiotics. The bacterial genera most commonly used as probiotics include *Bacillus*, *Lactobacillus*, *Enterococcus*, *Bifidobacterium*, and *Streptococcus*. Probiotics influence the gut by one or a combination of mechanisms, including that they modulate the host immune system, provide energy via SCFA production, and influence gut structure, integrity, and function. Probiotics also directly influence other bacteria, including pathogens, by production of metabolites and antimicrobial compounds, occupation of ecological niches within the gut to competitively exclude colonization of other bacteria, and by lowering the luminal pH. Some probiotic bacteria attach to receptors on enterocytes and activate Toll-like receptors (TLRs), leading to the induction of cytokine expression (5).

An important application of prebiotics and probiotics in layers is to reduce the colonization of pathogenic bacteria. Gut pathogens, such as *Salmonella* and *Campylobacter*, cause clinical diseases in many animals and humans. To trigger an inflammatory response, *Salmonella* internalizes into enterocytes and survives within macrophages and M cells (6). Laying chicks infected with *Salmonella* show higher viable counts in the ileum, cecum, and colon than in the crop (7). Unlike mammals, adult laying chickens colonized with nontyphoidal *Salmonella* spp. generally show no clinical signs; although mucoid and blood-tinged feces may be occasionally present (8). However, poultry-specific *Salmonella* serotypes, such as *Salmonella enterica* serovar Gallinarum and *Salmonella enterica* serovar Pullorum cause clinical diseases in chickens (9). The three main species of *Campylobacter* that cause health and food safety problems in poultry are *Campylobacter jejuni*, *Campylobacter coli*, and *Campylobacter hepaticus*. Among them, *C. hepaticus* has attracted recent attention because it has been found to cause spotty liver disease in laying chickens (10). In *C. hepaticus*, gene clusters associated with stress response, sialic acid modification, glucose utilization, and hydrogen metabolism are implicated in its pathogenicity (11). Chickens infected with *C. jejuni* and *C. coli* are often asymptomatic but still cause a potential threat to public health. *In vitro* study suggests that *Campylobacter jejuni* establishes its niche in epithelial cells via mechanisms involving serine protease HtrA (12). In laying chickens, prebiotics and probiotics have been used to reduce *Salmonella* and *Campylobacter* colonization in the gut (13–15). Therefore, the positive manipulation of the gut microbiota is a useful approach to improve food safety and control avian diseases such as spotty liver disease. Even though researchers and consumers generally accept the health benefit claims of prebiotics and probiotics, the underlying molecular mechanisms of action are not fully understood. An in-depth investigation of the underlying principles of the action of prebiotics and probiotics will provide confidence to use such supplements for therapeutic purposes. The objective of this review is to survey and summarize findings from the existing literature on gut microbiota in laying chickens as well as the use of prebiotics and probiotics for improving gut health and food safety in laying chickens and make recommendations for some of the key areas of focus for future work.

The composition of chicken gut microbiota and its role in gastrointestinal health. The composition of gut microbiota in laying chickens varies among the functionally different segments of the gastrointestinal tract, reflecting their different

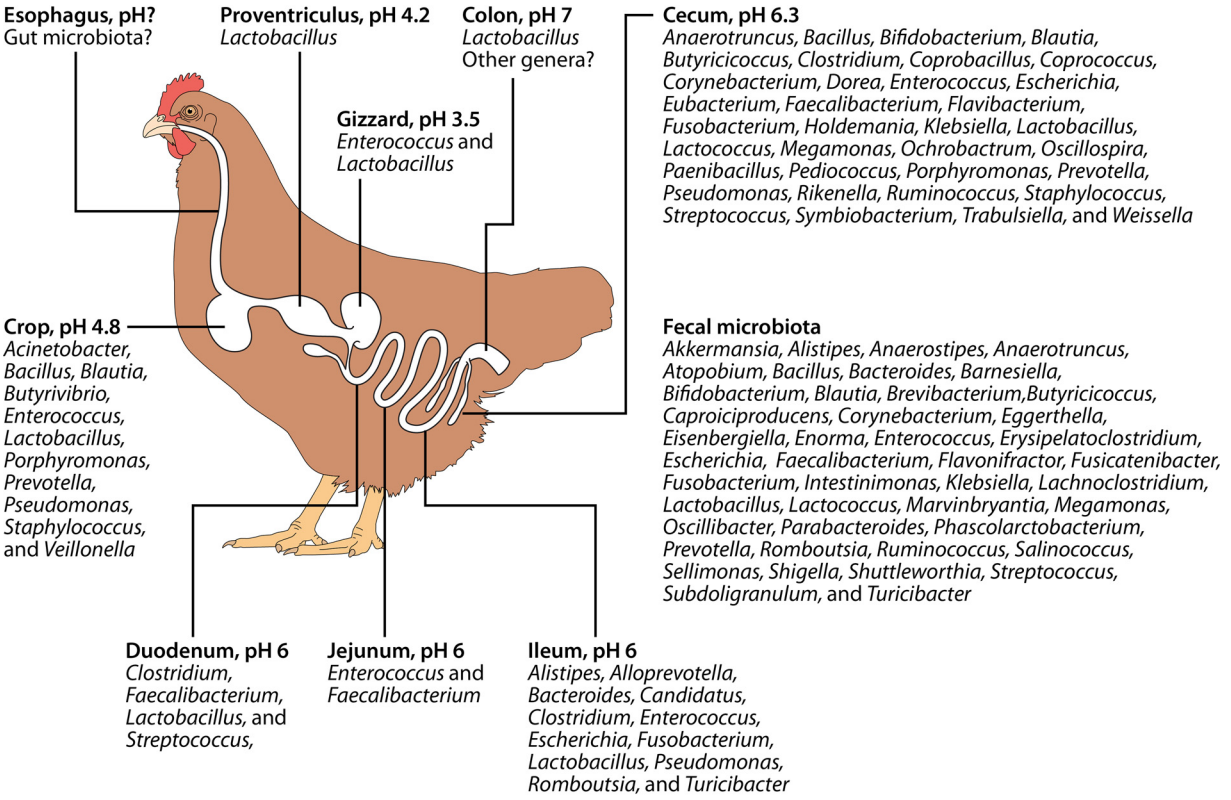

FIG 1 Composition and diversity of gut microbiota vary in different segments of the gut in chickens. The microbial composition and diversity are affected by multiple factors including host genetics, age, diet, and rearing conditions. The most common microbial genera depicted here may not be complete, as there is not enough information on individual gut segment microbiota in laying chickens. The information regarding genera present within each segment was collected from a range of relevant literature cited in the text.

physiochemical microenvironments (Fig. 1). The compartment pH, redox potential, growth substrates, antibacterial secretions, and metabolites from host and microbiota influence the colonization efficiency of microbes in the gut segments. The proximal segments of the gut are characterized by low pH, which strongly selects acid-tolerant bacteria and limits the growth of most pathogens (16). The crop is dominated by *Blautia*, *Lactobacillus*, *Bacillus*, *Pseudomonas*, *Enterococcus*, and *Staphylococcus*, while in ceca, in addition to the above, other bacteria such as *Faecalibacterium*, *Bifidobacterium*, *Clostridium*, and *Ruminococcus* are also abundant (17, 18). In the ceca of mature laying chickens, the representative microbial communities at the phylum level, in order of their typical abundance, are *Firmicutes*, *Bacteroidetes*, *Proteobacteria*, *Actinobacteria*, *Deferribacteres*, *Fusobacteria*, *Verrucomicrobia*, *Synergistetes*, and *Lentisphaerae* (19).

Gut microbial communities produce a range of metabolites that are involved in host functions, such as energy sources, cell to cell communication, and immune system regulation (Table 1). SCFAs and tryptophan catabolites affect host-microbiota cross talk (1). Microbial communities are able to metabolize dietary tryptophan into indole and its derivatives. Many indole derivatives, such as indole-3-acetaldehyde, indoleacrylic acid, indole-3-acid-acetic, and indole-3-aldehyde, act as aryl hydrocarbon receptor (AhR) ligands and modulate local and distant host functions that include epithelial barrier physiology and immune system homeostasis (1). The *Clostridiaceae*, *Ruminococcaceae*, and *Lachnospiraceae* contain diverse gene complements that encode enzymes involved in carbohydrate metabolism (20). The *Ruminococcaceae* are enriched in xylanase and cellulase genes, while both the *Ruminococcaceae* and *Lachnospiraceae* produce α-glucosidases and both α- and β-galactosidases (20). Members of the *Lachnospiraceae* and *Ruminococcaceae* families can cleave cellulose and hemicellulose to release sugars for utilization by both microbes and host; therefore, *Lachnospiraceae* and *Ruminococ-*

TABLE 1 Microbial metabolites and their functions in host gut and systemic health[a]

Gut metabolite	Microbe(s) involved	Target(s) of metabolite	Effect(s) on gut health	Reference(s)
Acetate	*Faecalibacterium*, *Anaerostipes*, *Eubacterium*, *Lactobacillus*	Activates GLP1, GPR41 and GPR43; activates MAPK pathway; energy substrate	Provides energy for enterocytes; cosubstrate for butyrate production; increases mineral absorption; inhibits growth of pathogens; regulates T cells in ceca; attenuates inflammasome activation	78, 79
Propionate	*Escherichia coli*, *Propionibacterium*, *Roseburia inulinivorans*, *Negativicutes*, *Bacteroides*, *Selenomonas ruminantium*, *Lactobacillus*, *Akkermansia*	Activates GLP1, GPR41, GPR43; upregulates leptin, peptide YY, and glucagon-like peptide	Antilipogenic and anti-inflammatory; provides energy for enterocytes; increases satiety	79, 80
Butyrate	*Lachnospiraceae*, *Ruminococcus*, *Bacteroides*, *Lactobacillus*, *Faecalibacterium*, *Eubacterium*	Activates GLP1; inhibits histone deacetylase; suppresses NF-κB pathway	Inhibits histone deacetylase; modulates immune system	79, 81
Succinate	*Prevotella copri*, *Veillonella parvula*, *Phascolarctobacterium succinatutens*	Activates SUCNR1	Proinflammatory; intermediate in propionate production	82
Tryptamine	*Bacteroides thetaiotaomicron*, *Ruminococcus gnavus*, *Clostridium sporogenes*	Activates epithelial 5-HT_4R to increase cAMP level in gut	Barrier functions; immune system modulation	83
Indole	*Eubacterium hallii*, *Clostridium bartlettii*, *Lactobacillus*, *Bacteroides fragilis*, *Parabacteroides distasonis*, *Bifidobacterium longum*, *Escherichia coli*	Modulates glucagon-like peptide 1 secretion; activates AhR and PXR in gut	Improves host metabolism; maintains host-microbe homeostasis; reduces inflammation and improves mucosal barrier function	84, 85
10-Hydroxy-*cis*-12-octadecenoate	*Lactobacillus plantarum*	Activates Nrf2 and GPR40; inhibits ERK phosphorylation	Anti-inflammatory; maintains intestinal barrier function; reduces obesity	86
Conjugated linoleic acid	*Lactobacillus casei*	Modulates cytokine production; activates PPARα and PPARγ; inhibits cyclooxygenase and lipoxygenase	Anti-inflammatory; reduces pathogen growth; reduces obesity	87
Bacteriocins	Multiple bacteria	Targets cell wall of pathogens; inhibits pathogen DNA gyrase-mediated DNA supercoiling; blocks aminoacyl-tRNA binding to the 50S ribosome	Inhibits the growth of pathogenic bacteria	70, 88
Vitamins K2 and B-complex	Multiple species of bacteria, including *Lactobacillus*, *Bifidobacterium*, *Bacteroides*, *Enterobacter*, *Serratia*, *Enterococcus*, *Lactococcus*	K2 acts as a cofactor for γ-glutamyl carboxylase; B-complex acts as a cofactor for multiple enzymes	Cell metabolism; role in clotting; anticoccidiostate; regulates immune system	89–91

[a]The gut metabolites mentioned in this table have been studied in nonchicken models. In chickens, mainly SCFAs have been studied, showing various dietary effects on their production. The molecular targets and functions of the gut microbiota metabolites in chickens need to be investigated for the broader role of beneficial microbes in health and disease. It should also be noted that gut effects reported in the table at a genus level refer to unidentified species of the genus and do not imply that the effect is existing in all species of that genus. AhR, aryl hydrocarbon receptor; ERK, extracellular signal-regulated kinase; GLP1, glucagon-like peptide 1; GPR41, G protein-coupled receptor 41; MAPK, mitogen-activated protein kinase; Nrf2, nuclear factor erythroid 2-related factor 2; PPARα, peroxisome proliferator-activated receptor alpha.

caceae may perform better than *Clostridiaceae* in degrading plant materials for the production of organic acids that are used by the host as energy sources (20). Future research could focus on investigating the hypothesis that the formation of gut microbial metabolites shapes the integrity of the epithelium and can be manipulated to improve gut barrier function in laying chickens.

Rearing conditions and flock age affect the gut microbiota. Rearing conditions and host-related factors, such as production system, sex, age, breed, and feed, may have profound effects on the development and composition of gut microbiota. However, rigorous analysis cannot be made, as there is not much literature available on the effects of these conditions on gut microbiota composition and diversity in layers. Correlations have been noted between gender, genotype, age, and body composition and the abundance of a number of microbial genera. For example, *Lactobacillus*, *Lactococcus*, and *Bifidobacterium* were found to be more abundant in low-body-weight laying chickens (21). The gut microbiota develops rapidly from day 1 to 3, and around day 7, most of the organisms that are found in the mature microbiota are already present, although the relative numbers tend to fluctuate for several weeks before stabilizing (22). After 2 weeks posthatch, *Ruminococcus* and *Oscillospira* increase substantially while the representation of *Enterococcus* is reduced (22). Compared with week 8 of chicken age, at week 30 *Firmicutes* and *Bacteroidetes* become more abundant in the gut (23). Assessing the effect of age (week 1 to 60) on the composition of gut microbiota in laying chickens, *Proteobacteria*, *Firmicutes*, and *Bacteroidetes* formed the vast majority of microbiota across all age categories (24). This shows that Gram-negative bacteria dominate the gut at an early age while *Firmicutes* become more prominent in the later age of the laying cycle of hens.

As chickens get older, the integrity of the gut mucosal system is compromised due to changes in the composition of the gut microbiota. The production system affects the development and composition of gut microbiota with a higher abundance of microbial genera involved in amino acid and glycan metabolic pathways in free-range compared with that in cage laying chickens (25). The abundance of *Bacteroidetes* is lower in cage birds, while the abundance of *Firmicutes* is higher in free-range birds (25). This shows that rearing conditions shape the composition of the gut microbiota. Further study of the natural development of gut microbiota in layers should be aimed at analysis of the effects on growth performance, egg production traits, and resistance to pathogen infection. Given that the production of free-range eggs is increasing globally, future studies on the development of gut microbiota should include a comparative analysis of hens raised in cage, free-range, and barn systems and the role of range soil microbiota in the modulation of chicken gut health. It could be hypothesized that the gut pathogens, once introduced, will persist for a long period, thereby affecting the composition and diversity of gut microbiota in hens during production.

Difference in the composition and diversity of gut microbiota in broilers and layers. The phylogenetic composition of microbiota typically found in various intestinal segments of broilers is well characterized (reviewed in references 26 and 27). However, in layers, there is only limited literature available on the composition of microbiota in the gut. The environmental conditions and physiological functions of the gut vary along its length, and these differences are reflected in different populations of microbial communities in various segments of the gut. The gut microbiota is more complex and richer in layers than in broilers (28). Fundamental differences in the composition of gut microbiota between broilers and layers have been reported. For example, using a $>$0.1% abundance threshold for microbiota analysis, *Cytophaga*, *Thermobaculum*, *Geobacillus*, *Desulfovibrio*, *Cyclobacterium*, *Caldicellulosiruptor*, and *Caulobacter* were present in the ceca of indigenous Indian layers but were not detected in broilers (3). It is evident from literature that many bacterial genera are common between broilers and layers; however, differences exist in the abundance and richness of individual genera due to differences in the energy requirements of broilers and layers. Given that broilers and layers have major differences in their physiology, husbandry, feeding practices, and life

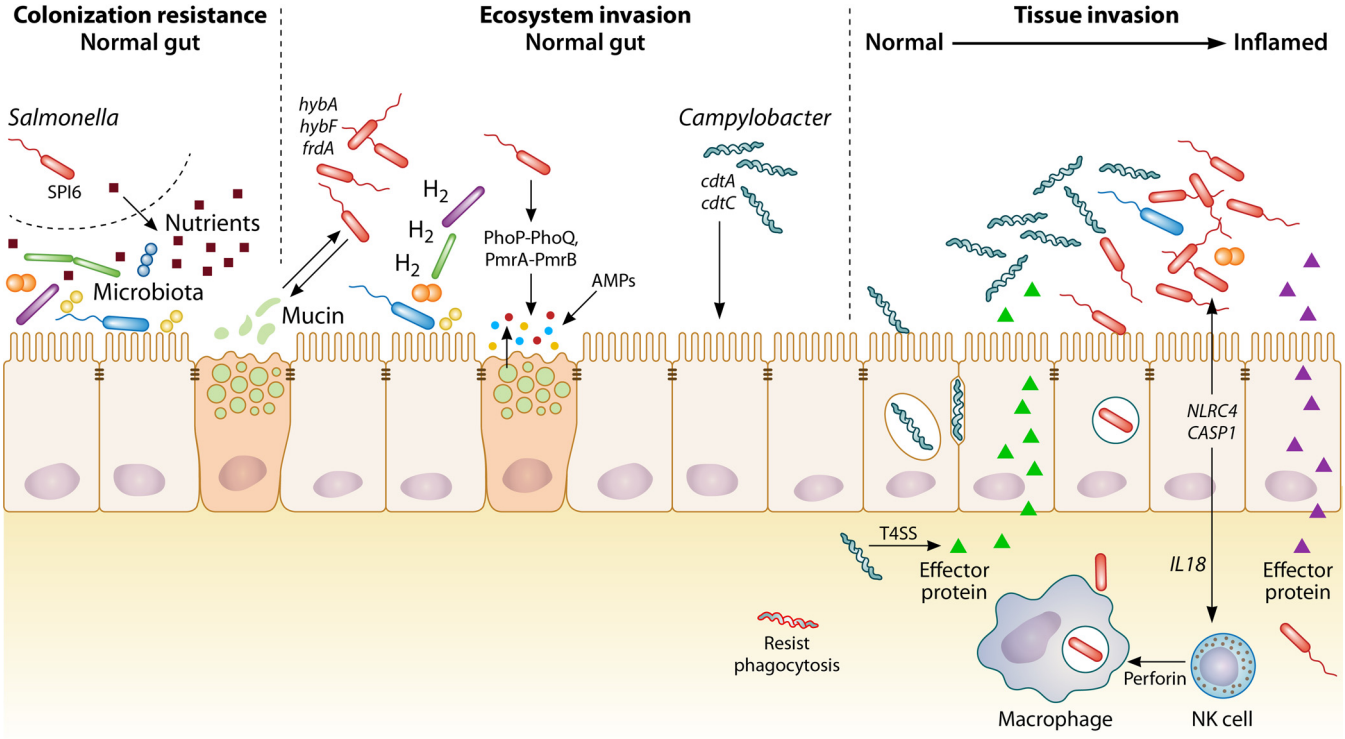

FIG 2 Generalized mechanisms of colonization by foodborne pathogens in the gut. Pathogens use various metabolic pathways to overcome the resident gut microbiota for establishing a niche in the gut. AMPs, antimicrobial peptides; *cdtA*, cytolethal distending toxin subunit A; *hybA*, hydrogenase-2 electron transfer unit; *frdA*, fumarate reductase subunit; T4SS, type IV secretory system.

span, it is difficult to compare their gut microbiota. Moreover, the endocrine changes at the onset of lay may potentially influence the gut microbiota in laying hens. Future research could test the hypothesis that differences in the composition of gut microbiota in each gut segments exist between broilers and layers reared in the same or different production systems and could explore the functional consequences of different microbiota compositions.

***Campylobacter* and *Salmonella* colonization in the chicken gut.** Egg-based products are among the leading causes of foodborne outbreaks of *Salmonella* infection. Gut dysbiosis is a microbial imbalance that results in the overgrowth of pathogenic bacteria and can lead to systemic infection (Fig. 2). The resident gut microbiota produces metabolites that inhibit the colonization of pathogenic bacteria. The efficient utilization of available nutrients by microbiota depletes the metabolic niches for pathogenic bacteria, such as *Salmonella* and *Campylobacter*. The resident gut microbiota can outcompete pathogens by saturating binding sites on gut epithelium that result in competitive exclusion. The host epithelial cells sense pathogen-associated molecular patterns (PAMPs) and thus boost the secretion of mucus, immunoglobulins, and antimicrobial peptides (AMPs). However, through the use of hyb hydrogenase enzymes, *Salmonella* can grow by consuming molecular H_2 secreted by microbiota (29). Once *Salmonella* establishes a colonization niche, it regulates the virulence genes vital for multiplication in the lumen and invasion into the host cells. The mechanisms of different host gut colonization by *Salmonella* and *Campylobacter* are different, as unlike *Campylobacter* (type IV secretory system [T4SS]), *Salmonella* employs a type III secretory system (T3SS) for establishing a niche and internalization into organs. Both *Salmonella* and *Campylobacter* can translocate via the transcellular or paracellular routes by breaking down tight junctions. The pathogens also release effector proteins and toxins that facilitate their colonization and invasion.

Campylobacter is a microaerophilic pathogen that requires O_2, H_2, and CO_2 for its growth; however, under anaerobic conditions, *Campylobacter* spp. express several

virulence factors (30) necessary for cell invasion. *Campylobacter jejuni* and *Campylobacter coli* can colonize chickens, usually without serious pathogenic effects; however, they cause clinical disease in humans. *C. jejuni* is transmitted mainly horizontally (31) in hens; however, globally, there is limited evidence for the association of *Campylobacter*-contaminated table eggs with human campylobacteriosis. The prevalence of *C. jejuni* in the gut is affected by production system, with its incidence higher in free-range chickens (32), suggesting that a different pattern of host gut microbiota composition may influence its colonization. *C. hepaticus* causes spotty liver disease in laying chickens with great economic losses. Spotty liver disease is more common in free-range chickens, and the pathogen is present in different segments of the gut of the infected birds (33). Genes that encode effector molecules required for niche adaptation, virulence, gut colonization, and invasion have been tentatively identified in *C. hepaticus* (33). One interesting avenue worth exploring in the future is to investigate interactions within the gut microbiota and how *Salmonella* may influence the shedding levels of *Campylobacter*, possibly via the production of gut microbial metabolites, as in a mouse model, coinfection of *Salmonella enterica* serovar Typhimurium increased the virulence of *C. jejuni* (34).

The complex interactions among gut microbiota, *Salmonella*, *Campylobacter*, and host are not completely understood in chickens. In the nutrient-limited environment caused by the intestinal microbiota, *Salmonella* uses specific metabolic traits for the utilization of compounds that are not metabolized by gut microbiota (Fig. 3). For example, *Salmonella* utilizes 1,2-propanediol, a product released during the fermentation of L-fucose. Most of the *Salmonella enterica* serovars (35) and *Campylobacter jejuni* (36) contain the fucose utilization operons that provide them a competitive advantage for the colonization of the host gut. Other bacteria, such as *Escherichia coli* and *Lactobacillus rhamnosus* GG, contain genes for fucose fermentation; however, their interactions with *Salmonella* and *Campylobacter* for consumption of fucose need to be investigated. It appears that inflamed epithelial cells present adhesion receptor sites that are exploited only by pathogenic bacteria. Reactive oxygen species generated by neutrophils during inflammation can react with endogenous thiosulfate to form tetrathionate. The ttrRSBCA locus on *Salmonella* pathogenicity island 2 confers *Salmonella* Typhimurium the ability to use tetrathionate as a terminal electron acceptor in anaerobic respiration (6). This confers a growth advantage to *Salmonella*, as it can use ethanolamine as a carbon source in the presence of tetrathionate (37). Under anaerobic conditions and in the presence of tetrathionate, 1,2-propanediol can serve as an energy source for *Salmonella* Typhimurium (35).

With the proliferation of *Salmonella* Typhimurium, the T3SS triggers inflammatory host responses that shift competition in favor of the pathogen (38); however, the exact mechanism in chicken is not known. The *Salmonella* Typhimurium Tat (twin-arginine translocation) system contributes to intestinal infection by facilitating colonization of the gut of mammals (39); however, its role, if any, in the gut of chickens needs to be confirmed, as Tat-deficient mutants of *Salmonella enterica* serovar Enteritidis did not influence cecal colonization in Leghorn chickens (40). In this system, two Tat-exported enzymes, peptidoglycan amidase AmiA and AmiC, are responsible for the Tat-dependent colonization. *Salmonella* employs a wide range of metabolic strategies for surviving and establishing a niche in the host gut that seems to be different between mammals and chickens. Future research could focus on understanding the role of resident gut microbiota and microbial metabolites in response to *Salmonella* infection in chickens, as they do not always develop clinical disease.

The use of probiotics and prebiotics in layer chickens for gut health. Diets supplemented with probiotics have been reported to significantly improve bird performance in terms of egg production and egg quality (41, 42). Probiotics improve the ecosystem of the gut in layers by balancing many of the microbial genera. For example, using culture medium as a method of quantitation, *Bacillus subtilis* increased the counts of bifidobacteria and lactobacilli and decreased clostridia and coliforms (41). Probiotic

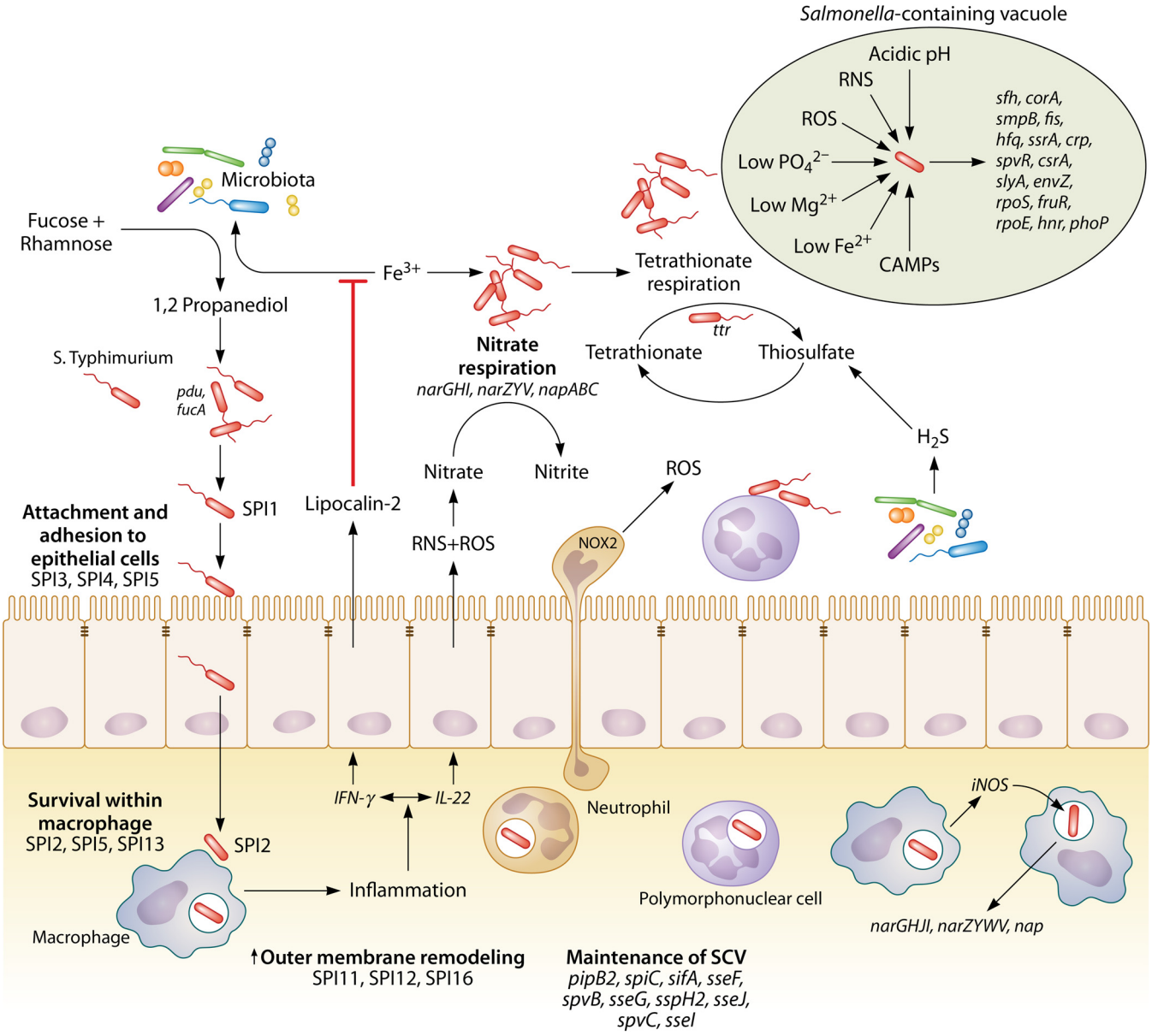

FIG 3 Generalized model shows that intestinal inflammation triggered by *Salmonella* Typhimurium results in its proliferation. *Salmonella* Typhimurium, once in the lumen of the gut, upregulates SPI1 to trigger intestinal inflammation. As a result, the released chemokines trigger the release of reactive nitrogen species (RNS), reactive oxygen species (ROS), and lipocalin-2 from the enterocytes. In turn, the lipocalin-2 blocks resident gut microbiota growth leading to a burst of *Salmonella* Typhimurium. This growth is further helped by the *Salmonella* Typhimurium nitrate and tetrathionate respiration strategies maintaining its continuous division in the lumen and tissue invasion. To protect itself from the inducible nitric oxide synthase encoded by the infected macrophages, *Salmonella* Typhimurium upregulates the membrane-bound *narGHJI*, *narZYWV*, and periplasmic *nap* nitrate reductases that could use NO_3^- as a respiratory substrate. Inside the host cell in a *Salmonella*-containing vacuole, *Salmonella* faces various stresses induced by acidic pH, RNS, ROS, reduced concentrations of phosphate, magnesium and iron, and cationic antimicrobial peptides (CAMPs). In response, *Salmonella* upregulates various regulons vital for its survival inside the vacuole. In chickens, some of these pathways may differ as *Salmonella* infection does not always result in the development of the clinical disease.

and synbiotic supplementation restored the gut ecosystem disrupted by *Salmonella* Typhimurium and increased the production of butyrate (43, 44). A *Pediococcus acidilactici* strain reduced the cholesterol level in egg yolk and improved tibial bone mineralization (42). An *Enterococcus faecium* strain and fructo-oligosaccharides significantly reduced serum cholesterol level in chickens and improved egg quality (45). Some probiotics have shown to lower pathogenic bacterial load, improve gut microbiota balance, and enhance the gut mucosal immune system (Table 2).

The use of prebiotics in layers has shown promising results for improving the population of certain beneficial bacterial genera in the gut. For example, a prebiotic

TABLE 2 Probiotics used for gut health in layer chickens

Probiotic	Model system	Disease type or model	Effect on gut microbiota	Function(s)	Reference(s)
Lactobacillus reuteri LM1	Layer	*Brachyspira pilosicoli*-induced gut inflammation	Microbiota was not investigated	Reduced colonization of *B. pilosicoli*	92
Lactobacillus salivarius CTC2197	Layer	*S.* Enteritidis-induced gut inflammation	Microbiota was not investigated	Reduced colonization of *Salmonella*	65
Multistrain probiotic[a]	Layer	*S.* Enteritidis-induced gut inflammation	Increased the population of *Pediococcus* and *Lactobacillus*	Reduced colonization of *Salmonella* in ceca	93
Lactobacillus suspension or *Lactobacillus plantarum*	Layer	*C. jejuni*- and *S.* Enteritidis-induced infections	Microbiota was not investigated	Gut colonization resistance to *C. jejuni* enhanced; no significant effect on *Salmonella* load reduction in organs; upregulated *IL-6*, *IL-10*, and *TLR4* in ileum	94, 95
Bacillus cereus var. toyoi (Toyocerin)	Layer/broiler	*S.* Enteritidis-induced enteritis	Microbiota was not investigated	Reduction in cecal *Salmonella* load	96
Bacillus subtilis PY79	SPF chicks	*S.* Enteritidis- and *Clostridium perfringens*-induced enteritis	Microbiota was not investigated	Reduction in bacterial load in gut	97
Citrobacter diversus, *Klebsiella pneumoniae*, and *Escherichia coli*	Layer	*C. jejuni* colonization	Microbiota was not investigated	Reduced *C. jejuni* load in ceca	98
Cecal culture	Layer	*C. jejuni* colonization	Microbiota was not investigated	Reduced *C. jejuni* load in ceca	15
Bacillus subtilis B2A	Layer	*S.* Gallinarum challenge	No significant effect on *Lactobacillus* and *Escherichia* populations in gut	Reduced *Salmonella* load in gut	99
Bacillus subtilis and *Bacillus amyloliquefaciens*	Layer	*S.* Typhimurium challenge	Restored abundance levels of *Christensenellaceae* R7 group, *Lachnospiraceae* UCG010, and *Ruminococcaceae* UCG005	Reduced overall load of *Salmonella* in feces	43
Multistrain probiotic[a]	Layer	*S.* Typhimurium challenge	Increased the abundance of *Ruminococcus*, *Trabulsiella*, *Bifidobacterium*, *Holdemania*, and *Oscillospira*	No significant reduction in *Salmonella* load in ceca	44

[a]Multistrain probiotics contain prebiotic component(s). There are not many research reports focused on the use of probiotics for their mechanistic effects on gut integrity and gut barrier functions in layers. Future research should focus on the strategic use of probiotics in layers for control of pathogens at different points of the production cycle. It is essential to select commercial probiotics that are shelf-stable, cost-effective, and feed-stable. There is substantial literature on the use of probiotics to control enteric pathogens in chicken meat birds; however, the long-term trials with laying chickens are limited due to costs and labor. Developing probiotic strains that can be used in place of in-feed antibiotic growth promoters for production of safer food products should be the target of future research. Characterization of *Faecalibacterium*, *Propionibacterium*, *Saccharomyces*, and *Roseburia* for improving gut health in laying chickens may be rewarding areas for future research. The increased abundance of *Faecalibacterium* in the gut of *Salmonella* Typhimurium-challenged laying hens that turned negative for *Salmonella* (43) suggests its potential role to be characterized as a probiotic candidate for gut health. The use of *Saccharomyces* in broilers has shown promising results on gut health. *Propionibacterium* exhibits anti-inflammatory properties and is used as a starter in the preparation of dairy products.

TABLE 3 The use of prebiotics for gut health in laying chickens[a]

Prebiotic	Model system	Disease type or model	Microbiota affected	Function(s)	Reference(s)
Inulin	Chicken macrophage HD11 cell line	*S.* Enteritidis-induced phagocytosis	Not applicable	Decreased viable cells of *S.* Enteritidis and production of IL-1β	100
Mannose-oligosaccharides	Layer	*S.* Enteritidis challenge	Increased *Bifidobacterium* and *Lactobacillus* in ceca	Reduced *S.* Enteritidis colonization in ceca	101
Fructo-oligosaccharides	Layer	*S.* Enteritidis challenge	No significant change in *Lactobacillus*, *Escherichia*, *Bifidobacterium*, and *Bacteroides* populations in ceca	Reduced *S.* Enteritidis colonization in ceca and feces; upregulated *TLR4* and *IFN-*γ in ileum	102, 103
Galacto-oligosaccharides	Layer	*S.* Enteritidis/*S.* Typhimurium challenge	Increased abundance of *Lactobacillus* and *Clostridium*	No significant effect on *Salmonella* load in ceca	14
Galacto-oligosaccharides	Layer	*S.* Typhimurium challenge	Increased abundance of *Lactobacillus* and *Christensenellaceae*	Increased clearance of *S.* Typhimurium from gut	104
Fructo-oligosaccharides/ lactose/mannose	Layer	*Campylobacter jejuni* challenge	Did not investigate	Reduced *C. jejuni* load in ceca	98

[a]A range of prebiotics can be used to improve gut health in laying chickens. As a result, food safety can be improved when there is less *Salmonella* and *Campylobacter* contamination in egg or egg products. Research is needed to investigate the effectiveness of these and other prebiotics for control of *Campylobacter hepaticus*, which is causing significant economic losses in the layer sector. It could be hypothesized that the continuous feeding of prebiotics improves host digestive functions and resistance to the colonization of pathogens in different gut segments.

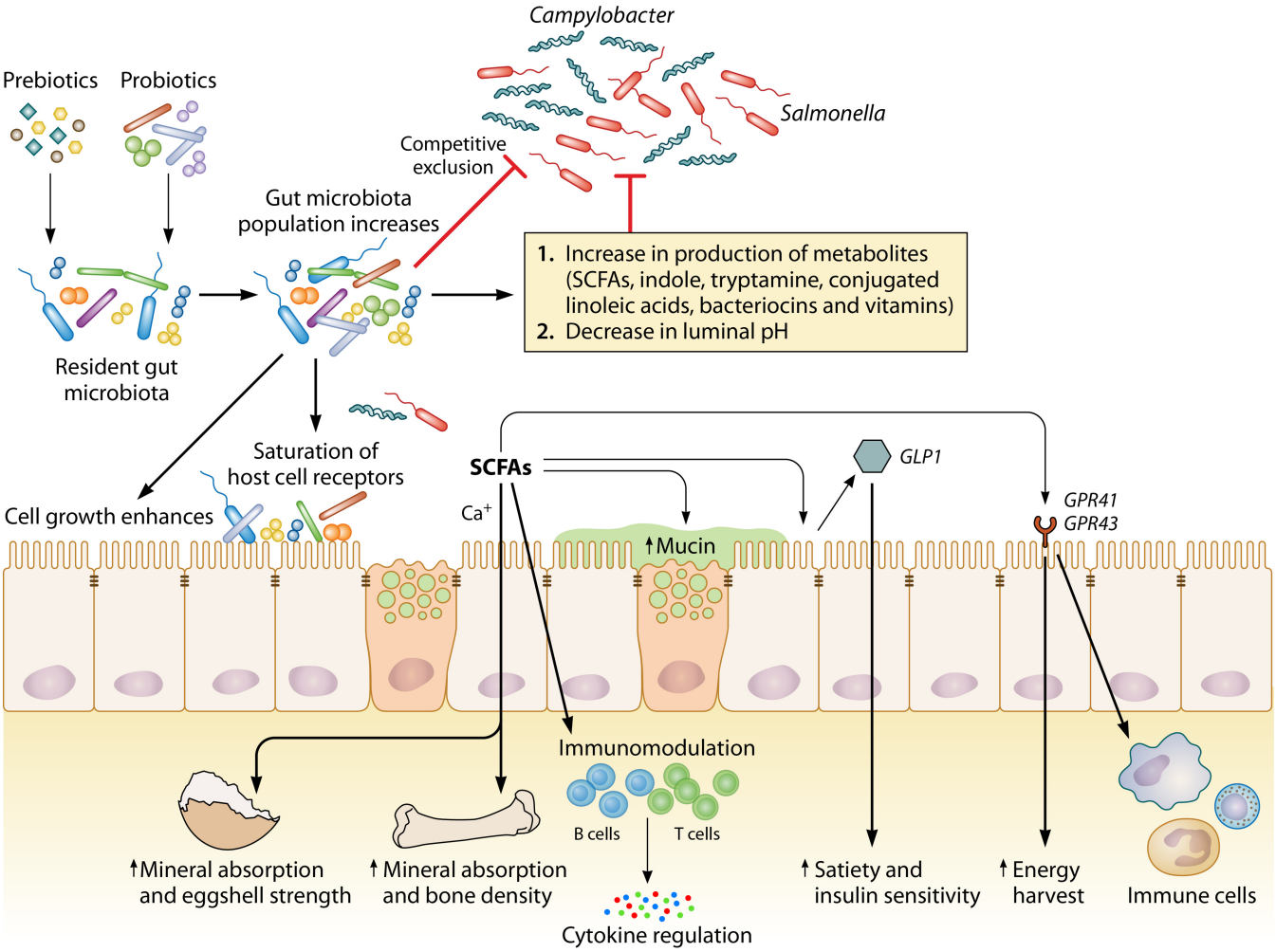

FIG 4 Overview of the mechanisms of action of probiotics and prebiotics. Prebiotics increase the population and functionality of certain resident gut microbiota that in turn competitively exclude pathogenic bacteria by mechanisms that include the production of microbial metabolites, mucin production, and modulation of the host immune system. Unlike prebiotics, probiotics are directly available in the gut for the desired functions. During the microbial fermentation, short-chain fatty acids (SCFAs) are produced that exert beneficial effects on the host involving various mechanisms. Regulation of the host immune system is influenced by the increased biomass and cell wall components of the bacteria. Depending upon the compositions of prebiotics and probiotics, they may result in the increase of certain genera of gut microbiota, leading to decreased microbial diversity. The decreased microbial diversity may or may not be useful depending upon the nature of the microbial community. Future research needs to address the mechanistic understanding of the interactions of prebiotics and probiotics with the composition of gut microbiota and its metabolites in laying chickens in different production systems.

increased the abundance levels of *Lactobacillus* and *Olsenella* and the expression of genes in microbial communities associated with propanoate and butanoate metabolism in the gut of layers (46). The prebiotics used for the control of *Salmonella* and *Campylobacter* in layer production are summarized in Table 3. Table 3 shows that the prebiotics produced variable results in terms of reducing pathogen load in the gut and priming the host immune system.

Mechanisms of action of probiotics. The functions of probiotics are achieved through bacteria-bacteria and host-bacteria interactions (Fig. 4). The bacteria-bacteria interactions result in the production of SCFAs, modification of redox potential, the production of antimicrobial compounds, competition for epithelial receptors, quorum sensing, and production of ecosystems unsuitable for pathogen colonization. Probiotic strains of bacteria have cell walls that contain components, such as capsular polysaccharide, peptidoglycan, teichoic acids, and lipoproteins (47). These molecular components represent microbe-associated molecular patterns (MAMPs) that are recognized by specific host intestinal mucosal pattern-recognition receptors (PRRs) that function to prime the immune system to suppress pathogens (47).

Immunomodulatory action. Probiotics influence immune functions of the host by several pathways. *In vitro* studies have shown that maturation of human dendritic cells and production of interleukin-10 (IL-10) can be induced by the binding of *Lactobacillus reuteri* and *Lactobacillus casei* to CD209 (48). Other probiotics, such as *Lactobacillus acidophilus* NCFM, *L. rhamnosus* GG, and *Lactobacillus plantarum* WCFS1 have the potential to induce signaling via Toll-like receptors (TLRs) through the production of lipoteichoic acid that contains di-acyl or tri-acyl glycolipids (47, 49). Generally, the interactions between probiotics and host cells lead to the production of natural and antigen-specific antibodies, signal induction via TLRs, and regulation of T cells and cytokines. For example, in an *in vitro* study of chicken splenic and cecal tonsil cells, *L. acidophilus* and *Lactobacillus salivarius* induced Th1 and cytokine anti-inflammatory responses, respectively (50). *Lactobacillus* DNA induced *STAT2*, *STAT4*, *IL-18*, *IFN-*γ, *MyD88*, and *IFN-*α gene expression in chicken cecal tonsil cells (51). In broiler chicks challenged with *Salmonella* Typhimurium, a *Lactobacillus*-based probiotic lowered the expression levels of *IL-1*β, *IL-6*, and *IFN-*γ and increased the expression level of *IL-10* in cecal tonsils (52). Gene expression of *IFN-*γ was significantly reduced following probiotic feeding of chickens infected with *Salmonella* (53). There seems to be a synergy between vaccines and probiotics on the gut immune system that can modulate the clearance of pathogens, as coadministration of *L. reuteri and Anaerosporobacter mobilis* with an N-glycan-based vaccine resulted in lower gut colonization by *Campylobacter jejuni* and improved immune response (serum IgY antibodies) and gut microbiota composition in broilers and specific-pathogen-free (SPF) leghorns (54). These results show that probiotics can be used both in prophylactic and therapeutic ways to prime cytokine expression to modulate the host immune system against pathogens. Future research needs to focus on a mechanistic approach to understand the roles of probiotics in regulating NF-κB and mitogen-activated protein kinase (MAPK) pathways in disease conditions in laying chickens. Future research should expand on the finding that the immune response elicited by probiotic bacteria varies with the bacterial strains, and, therefore, there is a need to identify a probiotic strain suitable for boosting the host immune response in the presence of live vaccine strains of gut pathogens, such as *Salmonella aroA*-based vaccines.

Regulation of tight junction proteins. The integrity of the gut epithelium is maintained by the formation of tight junction protein complexes between cells. Tight junctions are multiprotein complexes that regulate the movement of ions and inhibit the translocation of pathogenic bacteria between gut epithelial cells. Changes in the tight junction proteins disrupt the intestinal mucosal barrier, thereby allowing the movement of pathogens across the epithelia. Probiotics influence barrier function by the direct inhibition of pathogens, by enhancing the synthesis of tight junction proteins, or by rearrangement of tight junction protein conformation (47). Studies in various animals have shown the beneficial effects of probiotics on the regulation of tight junction proteins in health and disease conditions. For example, *Bifidobacterium infantis* in mice suffering from necrotizing enterocolitis stabilized tight junctions by increasing the expression of claudin 2, 4, and 7 and occludin in gut tissue (55). In broiler chickens injected with lipopolysaccharide (LPS) from *Escherichia coli*, *Bacillus subtilis* probiotic upregulated the expression levels of *JAM2*, occludin, *ZO1*, and *MUC2* (56). The stabilized tight junction proteins increased host epithelial barrier protection against pathogen invasion into internal organs. We suggest that research should focus on confirming the role of various probiotic strains in stabilizing the regulation of tight junction proteins in laying chickens challenged with foodborne pathogens, as laying cycles induce stress that may result in the increased chances of pathogens translocation in the gut.

Mucin production. The gut epithelium consists of enterocytes, Paneth cells, goblet cells, M cells, and neuroendocrine cells, while the lamina propria contains immune cells, such as lymphocytes, macrophages, plasma cells, and dendritic cells. The enterocytes are mainly involved in nutrient absorption; the Paneth cells secrete AMPs, while the

goblet cells produce mucin. Mucin is a site for bacterial adhesion with subsequent competition between commensal and pathogenic bacteria. The gut microbiota interacts with mucin on several different levels; it influences mucosal cell proliferation and mucin synthesis and degradation (57). Some probiotics promote the development of goblet cells and increase the production of mucin (58). In a mouse model, it has been shown that *Bifidobacterium* adheres to the intestinal mucus and secretes γ-aminobutyric acid as a metabolite that upregulates *MUC2* for modulating the goblet cell functions with a net increase in mucin production (59). In broilers, supplementation of *Lactobacillus*-, *Bifidobacterium*-, and *Enterococcus*-based probiotics increased the goblet cell cup area in the gut and significantly upregulated the expression of *MUC* and increased the production of mucin glycoprotein (58). It appears that the induction of mucin production in the gut is strain specific, as in broilers, diet supplemented with *Bacillus subtilis* resulted in higher expression of *MUC2* and increased goblet cell density and jejunal villi height; however, the *Enterococcus*-, *Bifidobacterium*-, and *Lactobacillus*-based supplemented diet resulted only in increased goblet cell density and jejunal villi height (60). Alteration of the composition of gut microbiota can result in mucin degradation during infection. For example, in mice infected with *Citrobacter rodentium*, the microbiota was dominated by bacterial species that degrade mucins (61). Future research in layers could investigate the hypothesis that strategic feeding of probiotics and prebiotics can restore the disruption of mucin production by gut pathogens, such as *Salmonella* and *Campylobacter*.

Competition for adhesion sites. Some probiotic bacteria adhere to the apical brush borders of enterocytes, possibly thorough proteinaceous adhesion-promoting factors present in probiotic bacteria. Competitive exclusion due to inhibition of adhesion of pathogens in the gut has been studied using cell culture. For example, *L. acidophilus* inhibited the adhesion of *S*. Typhimurium to enterocyte-like Caco-2 cells (62). Some probiotic bacteria have strong aggregation properties that prevent pathogens from attachment to enterocytes. For example, in human uroepithelium, lipoteichoic acid of *Lactobacillus* inhibited the adherence of uropathogens (63). In *in vitro* conditions, multistrain probiotic bacteria competitively inhibited the attachment of *Salmonella* to human intestinal mucosa (64). *L. salivarius* CTC2197 seems to function through competitive exclusion to reduce *Salmonella* Enteritidis colonization in laying chickens (65). Although the competitive exclusion properties of probiotics have been studied and tested *in vitro*, their applicability and efficiency in colonization resistance to gut pathogens in chicken models are yet to be established. The most efficacious competitive exclusion products are complex bacterial mixtures derived from the ceca of healthy birds. Such products are difficult to standardize and quality control and, hence, are not acceptable in some markets. It would be desirable to find defined probiotic strains of bacteria that could perform as well as some of the undefined competitive exclusion products in reliably excluding foodborne pathogens and, thus, improve food safety in layers.

Bacterial metabolites. Probiotics produce a range of metabolites that include SCFAs, indole, tryptamine, bacteriocins, and vitamins (Table 1). The three most common SCFAs produced by gut microbiota are propionate, butyrate, and acetate. The SCFAs are an important energy source for enterocytes and induce the production of host AMPs. The AMPs are produced as a result of binding of SCFAs to G protein-coupled receptors (e.g., GPR41 and GPR43) to stimulate β-defensins and RegIIIγ (66). Propionate can restrict the growth of *Salmonella* Typhimurium through the disruption of intracellular pH homeostasis (67). SCFAs inhibit the growth of *Salmonella* when present in the dissociated form. For example, in a coculture at pH 5.8, SCFAs inhibited the growth of *Salmonella* Enteritidis (68). SCFAs also modify the expression of *Salmonella* virulence genes. For example, the expressions of SPI1 gene regulators (*hilA*, *hilD*, and *invF*) in *Salmonella* Typhimurium were significantly reduced by propionyl-coenzyme A (propionyl-CoA), a product of propionate metabolism (69). As a range of microbes produce SCFAs, we suggest that further investigation of the efficacy of specific probi-

otic strains for production of gut metabolites in the presence and absence of pathogens would be desirable.

Bacteriocins production. Bacteriocins are ribosomally synthesized peptides with antimicrobial properties produced mainly by Gram-positive bacteria; however, certain Gram-negative bacteria can also produce them. The widespread occurrence of bacteriocin peptides in bacterial species of the gut microbiota (70) suggests their regulatory role in population dynamics of gut pathogens as shown for the bacteriocin produced by *L. salivarius* UCC118 in *Listeria*-infected mice (71). A potent bacteriocin-producing probiotic, *Enterococcus faecium* KH 24, resulted in low shedding levels of *Salmonella* Enteritidis in a mouse model (72). None of the available probiotic strains for feed supplementation in laying chickens have been tested for the production of bacteriocins *in vitro* and *in vivo*. Therefore, the prominent role that bacteriocin production may play in the effectiveness of some probiotics in laying chickens needs to be confirmed. Enhancing their synthesis through strategic feeding of probiotics in the rearing phase of layer production might confer resistance to pathogenic bacterial colonization soon after transportation to production housing systems.

Mechanisms of action of prebiotics. Prebiotics in feed specifically alter the abundance of bacteria within the gut microbiota. The action of prebiotics is exerted via these altered bacterial populations and the metabolites that are produced (Fig. 4). Therefore, we have avoided writing subsections on the mechanisms of action of prebiotics.

As nondigestible by host enzymes, the prebiotics reach the lower gut where they are available to the resident gut microbiota as nutrient. Genomic analysis of bifidobacteria and lactobacilli shows carbohydrate metabolic gene repertoires that are involved in fermentation (73). Fermentation releases sugars and SCFAs that lower the gut luminal pH (Fig. 4). Applying these mechanisms, xylo-oligosaccharides increase the number of lactobacilli in colon and *Clostridium* cluster XIVa in ceca (74). To enhance the growth of certain gut microbial community members, prebiotics act as a carbon and energy source for the growth of microbes, such as *Bifidobacterium longum*, *Bifidobacterium adolescentis*, *Lactobacillus fermentum*, and *Lactobacillus brevis* (75). Certain prebiotics inhibit the growth of pathogens in the gut by manipulating the mechanisms of pathogenicity. For example, *Salmonella* can bind to mannose via type 1 fimbriae, leading to colonization inhibition (76). The competitive exclusion is mainly achieved through the increased population of resident gut microbiota by saturating the available receptors on enterocytes; however, this property of the prebiotics has not been thoroughly investigated. Research is needed to understand the role of prebiotics in the development, composition, and diversity of microbial communities in different segments of the gut (including crop and gizzard) in the presence of pathogenic bacteria, as the current notion is that prebiotics are fermented mainly in ceca and colon. Research in laying chickens has confirmed that prebiotics possess immunomodulatory properties (Table 3) and may increase calcium transport in the gut for improving egg quality (77). Future research should focus on investigating the hypothesis that prebiotics improve shell quality and cuticle cover through the modulation of gut microbiota for increased mineral transport.

Conclusions and recommendations. The gut microbiota is associated with the health and performance of birds. The available literature suggests that the gut microbiota in newly hatched chicks passes through different stages of maturation. However, further research is required to explicitly understand the effects of age, rearing conditions, and stress factors on the development and maturation of microbiota in various segments of the gut of layers. The commercial life span of layers is substantially longer than that of broilers. Hence, studies are required to understand the development and maturation of microbiota in different segments of the gut in laying chickens during their commercial life span. Establishment of a successful niche by *Salmonella* and *Campylobacter* in a chicken's gut highlights their ability to switch onto various metabolic pathways that help them to overcome the host gut microbiota. Moreover, while *Salmonella* and *Campylobacter* are the main pathogens of concern in food safety, it is

likely that prebiotics and probiotics will continue to play a role in the control of these pathogens. The microbiota can be modified by in-feed supplementation of prebiotics and probiotics for improving gut health. We suggest that future research should focus on understanding the mechanistic interactions between prebiotics/probiotics, gut segment microbiota, and pathogens in order to improve avian health and reduce the use of in-feed antibiotic growth promoters.

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